REMARKS

I. Status of the Claims

Applicants thank Examiner Smith and Examiner Marshall for taking the time to discuss the pending Office Action in a telephone interview with applicants' representative. Amendment to claims 1, 10, and 11 were discussed.

Claims 1-24 are pending in the application. With this amendment, claims 2-9 and 12-24 are canceled, in response to the restriction requirement mailed April 20, 2002, without prejudice to pursuing the claims in a continuing application. Claim 1 is canceled in order to reduce the issues, without prejudice to pursuing the claims in a continuing application. Therefore, claims 10 and 11 are currently pending. Claims 10 and 11 have been amended. Claim amendments are for the purposes of improved clarity or consistency of claim language unless otherwise noted. No claim amendment should be construed as an acquiescence in any ground of rejection. No new matter has been added by this amendment. Support for the amendment to claims 10 and 11 can be found throughout the specification and, for example, on page 30, lines 11-20; page 57, Table 1; and page 78, lines 26-28. The amendment is necessary and was not earlier presented because it is in response to the new grounds of rejection set forth in the final Office Action mailed June 25, 2004. Since the amendment obviates the outstanding grounds of rejection as discussed below, reduces the number of issues, contains no new matter, and places the application in condition for allowance or better condition for appeal, the amendment should be entered.

Claims 10 and 11 are rejected under 35 U.S.C. § 112, first paragraph, for failing to comply with the written description requirement. Claims 1 and 10 are rejected under 35 U.S.C. § 112, first paragraph, for lack of enablement. Claims 1, 10, and 11 are rejected under 35 U.S.C. § 102(e)(2) as being anticipated by Capon et al. (U.S. Patent 6,103,521).

II. Oath or Declaration

The Examiner states that the oath or declaration is defective and requires a new oath or declaration in compliance with 37 CFR § 1.67(a). This corrected Declaration, revising the citizenship of Applicant Früh, is being filed herewith as a Supplemental Declaration.

III. Specification

The title of the invention has been amended to indicate the invention to which the claims are directed.

IV. Patentability under 35 U.S.C. § 112, first paragraph

Written Description

Claims 10 and 11 have been rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description requirement.

Applicants have amended claims 10 and 11 merely to clarify the claimed invention. The specification provides an adequate written description for a method comprising, in part, administration of a therapeutically effective amount of a compound that inhibits receptor tyrosine kinase c-Kit and administration of a therapeutically effective amount of a compound which compound is different from the compound that inhibits receptor tyrosine kinase c-Kit and that modulates KSHV replication, as in claim 10. The specification provides adequate support for a therapeutically effective amount of a compound that inhibits receptor tyrosine kinase c-Kit, for example, c-Kit inhibitors, STI 571, Ad/c-KitDN (dominant negative c-Kit protein), or antisense oligonucleotide to c-Kit. See specification, for example, page 50, line 1 to page 51, line 3; and page 55, line 1 to page 56, line 24. The specification further provides adequate support for a therapeutically effective amount of a compound which compound is different from the compound that inhibits receptor tyrosine kinase c-Kit and that modulates Kaposi's sarcoma herpesvirus (KSHV) replication. Such compounds include, for example, daunorubicin, doxorubicin, interferon alpha, retinoids, and taxol, and inhibitors PDTC, trans-retinoic acid, SB203580, CGRP[8-37], Ht-31, haloperidol, 8-aminoguanosine, t22, and BQ788. See specification, for example, pages 51-52 and Table 1. Applicants' claimed invention provides a method for inhibiting replication of KSHV comprising, in part, administration of a therapeutically effective amount of a compound that inhibits receptor tyrosine kinase c-Kit. The claimed method further provides administration of a therapeutically effective amount of a compound which compound is different from the compound that inhibits receptor tyrosine kinase c-Kit and that modulates KSHV replication, as described in the specification and as known in the art. Therefore, the compounds of the

claimed method are described in the specification. Applicants respectfully request that the rejection of claims 10 and 11 under 35 U.S.C. § 112, first paragraph be withdrawn.

Enablement

Claims 1 and 10 have been rejected under 35 U.S.C. § 112, first paragraph, for allegedly lacking enablement. The Office Action asserts that the specification does provide enablement for using several compounds in the method that were laboratory-tested, but does not provide reasonable enablement for the broad term "compound" as stated in claims 1 and 10. Applicants traverse the rejection.

The rejection of claim 1 has been rendered moot since applicants have canceled claim 1 to reduce the issues and without prejudice reserving the right to pursue the claim in a continuing application. Applicants maintain that claim 10 is fully enabled. Applicants' claimed invention is, in part, a method for inhibiting replication of Kaposi's sarcoma herpesvirus (KSHV) comprising administration of a therapeutically effective amount of a compound that inhibits receptor tyrosine kinase c-Kit. Applicants provide two examples of the claimed method, one using the compound STI 571, which inhibits several receptors, c-Kit, Abl, PDGF β receptor tyrosine kinase. Further studies indicated that c-Kit was the primary target of STI 571 that leads to inhibition of KSHV replication. Further definitive studies using the compounds Ad/c-KitDN, a dominant negative c-Kit protein, or an antisense oligonucleotide to c-Kit confirmed that the mechanism for inhibiting replication of KSHV was through inhibition of c-Kit signaling pathway. Applicants further provide examples of a method for inhibiting replication of Kaposi's sarcoma herpesvirus (KSHV) comprising, in part, administration of a therapeutically effective amount of a compound which compound is different from the compound that inhibits receptor tyrosine kinase c-Kit and that modulates KSHV replication. Such compounds include, for example, daunorubicin, doxorubicin, interferon alpha, retinoids, and taxol, and inhibitors PDTC, trans-retinoic acid, SB203580, CGRP[8-37], Ht-31, haloperidol, 8-aminoguanosine, t22, and BQ788. From these working examples, one skilled in the art using reasonable experimentation can identify methods for inhibiting replication of KSHV and receptor tyrosine kinase c-Kit. The method for inhibiting replication of Kaposi's sarcoma herpesvirus (KSHV) comprises administration of a therapeutically effective amount of a compound that inhibits receptor tyrosine kinase c-Kit

and administration of a therapeutically effective amount of a compound which compound is different from the compound that inhibits receptor tyrosine kinase c-Kit and that modulates KSHV replication. Claim 10 is enabled. Applicants respectfully request that the rejection of claim 10 under 35 U.S.C. § 112, first paragraph be withdrawn.

V. The Claims Are Patentable Over the Cited References

Claims 1, 10, and 11 have been rejected under 35 U.S.C. § 102(e)(2) as allegedly being unpatentable over Capon et al. (U.S. Patent 6,103,521; "Capon et al. reference"). Applicants traverse the rejection.

The rejection of claim 1 has been rendered moot since applicants have canceled claim 1 to reduce the issues and without prejudice reserving the right to pursue the claim in a continuing application.

The Capon et al. reference fails to teach or suggest a method as defined in claims 10 and 11, that is, a method for inhibiting replication of Kaposi's sarcoma herpesvirus (KSHV) comprising administration of a therapeutically effective amount of a compound that inhibits receptor tyrosine kinase c-Kit and administration of a therapeutically effective amount of a compound which compound is different from the compound that inhibits receptor tyrosine kinase c-Kit and that modulates KSHV replication. The examiner stated that the Capon et al. reference discloses multispecific chimeric receptors that include "proliferation signaling domains from tyrosine kinase growth factor receptors, including c-Kit." Contrary to the examiner's assertion, the Capon et al. reference does not show applicants' claimed invention, in part, a method for inhibition of KSHV replication occurs by administration of a therapeutically effective amount of a compound that inhibits receptor tyrosine kinase c-Kit. Rather the Capon et al. reference incorporates the c-Kit tyrosine kinase domain as a "proliferation signaling domain" *that induces cell proliferation*. See Capon et al. reference, Column 10, lines 43 to 62. In contrast to applicants claimed methods, the methods and compositions of the Capon et al. reference do not inhibit replication of KSHV by inhibiting receptor tyrosine kinase c-Kit.

The Capon et al. reference does not support the assertion that taxol inhibits replication of KSHV by inhibiting receptor tyrosine kinase c-Kit. The Capon et al. reference discloses taxol as a therapeutic agent, but does not disclose a therapeutically effective amount of a

compound that inhibits receptor tyrosine kinase c-Kit. Rather, taxol is one of the compounds of the claimed method which provides, in part, administration of a therapeutically effective amount of a compound which compound is different from the compound that inhibits receptor tyrosine kinase c-Kit and that modulates KSHV replication. The Capon et al. reference provides no indication of a relationship between taxol activity and inhibition of receptor tyrosine kinase c-Kit, much less a relationship between taxol activity and a method of inhibition of replication of KSHV by a compound that inhibits receptor tyrosine kinase c-Kit. Rather, as stated in applicant's specification, taxol inhibits KSHV replication by a mechanism other than inhibition of receptor tyrosine kinase c-Kit. See specification, for example, page 30, lines11-20, and page 78, lines 22-28. In further support, applicants submit a copy of an article entitled "Mechanism of Paclitaxel Activity in Kaposi's Sarcoma," Sgadari et al., in *Journal of Immunology*, 2000, **165**: 509-517. See Exhibit 1 attached. The article states in the Abstract that paclitaxel (taxol) is "a microtubule-stabilizing drug known to inhibit Bcl-2 antiapoptotic activity. ...Our results suggest that paclitaxel interferes with KS [Kaposi's sarcoma] by down-regulating Bcl-2 antiapoptotic effect." Therefore, claims 10 and 11 are novel in view of the Capon et al. reference because the Capon et al. reference fails to teach or suggest a method for inhibiting replication of Kaposi's sarcoma herpesvirus (KSHV) comprising administration of a therapeutically effective amount of a compound that inhibits receptor tyrosine kinase c-Kit and administration of a therapeutically effective amount of a compound which compound is different from the compound that inhibits receptor tyrosine kinase c-Kit and that modulates KSHV replication.

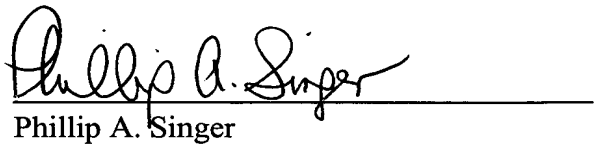
Since the claims patentably define over the Capon et al. reference, Applicants respectfully request that the anticipation rejection of claims 10 and 11 under 35 U.S.C. § 102(b) be withdrawn.

VI. Conclusion

In view of the foregoing, the application is now in condition for allowance. The prompt issuance of a formal Notice of Allowance is therefore requested.

If the Examiner believes a telephone conference would expedite allowance of this application, please telephone the undersigned at 206-332-1380.

Date: August 31, 2004



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Enclosures:
Supplemental Declaration
Exhibit 1: "Mechanism of Paclitaxel Activity in Kaposi's Sarcoma," Sgadari et al., in *Journal of Immunology*, 2000, **165**: 509-517

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Exhibit 1

Mechanism of Paclitaxel Activity in Kaposi's Sarcoma[1]

Cecilia Sgadari,* Elena Toschi,* Clelia Palladino,* Giovanni Barillari,* Davide Carlei,* Anna Cereseto,* Cristiana Ciccolella,* Robert Yarchoan,† Paolo Monini,* Michael Stürzl,‡ and Barbara Ensoli[2]*

Kaposi's sarcoma (KS) is an angioproliferative disease characterized by proliferation of spindle-shaped cells predominantly of endothelial cell origin, neoangiogenesis, inflammatory cell infiltration, and edema. At least in early stage, KS behaves as a reactive lesion sustained by the action of inflammatory cytokines and growth factors, has a polyclonal nature, and can regress. However, in time it can become monoclonal, especially in the nodular stage, evolving into a true sarcoma, likely in association with the increased expression of antiapoptotic oncogenes. We have recently demonstrated by immunohistochemical analysis that Bcl-2, a proto-oncogene known to prolong cellular viability and to antagonize apoptosis, is highly expressed in spindle cells and vessels of both AIDS-KS and classical KS lesions and that its expression increases with lesion stage. Paclitaxel, a microtubule-stabilizing drug known to inhibit Bcl-2 antiapoptotic activity and to be highly effective in the treatment of certain neoplasms, has recently been found to be active also in patients with advanced HIV-associated KS. In this report we investigated the mechanism(s) of paclitaxel activity in KS. By using a model of experimental KS induced by the inoculation of KS-derived spindle cells in nude mice and primary cultures of KS spindle cells, we found that paclitaxel promotes regression of KS lesions in vivo and that it blocks the growth, migration, and invasion of KS cells in vitro. Furthermore, paclitaxel treatment promoted apoptosis and down-regulated Bcl-2 protein expression in KS cells in vitro and in KS-like lesions in mice. Our results suggest that paclitaxel interferes with KS by down-regulating Bcl-2 antiapoptotic effect. *The Journal of Immunology,* **2000, 165: 509–517.**

Kaposi's sarcoma (KS)[3] is a multifocal angioproliferative disease characterized by the proliferation of spindle-shaped cells predominantly of vascular origin considered to be the neoplastic elements of KS, neoangiogenesis, inflammatory cell infiltration, and edema (1). KS is found in four clinic-epidemiological forms: "classic" or "Mediterranean" KS, "endemic" or "African" KS, "posttransplant" KS, and "AIDS-associated" KS (AIDS-KS). Although these forms show different geographical distribution and clinical course, they share common features. These include the histopathology of the lesions; the presence in lesions of high levels of inflammatory cytokines (IC), such as γIFN, TNF, and IL-1; and angiogenic molecules such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor, which mediate lesion formation (2–11). Recently a new viral agent termed human herpesvirus-8 (HHV-8) has been found in all forms of KS (12–16). This evidence suggests a common etiopathogenesis for all forms of KS.

Several experimental data indicate that at least in early stage KS is not a true sarcoma but an angio-hyperplastic-inflammatory lesion mediated by the above mentioned factors whose production may be triggered or enhanced by infection with HHV-8 (7, 17, 18). Virus reactivation, in turn, is induced by the IC increased in KS and in individuals at risk of KS (17). In this context the Tat protein of HIV can increase the frequency and aggressiveness of AIDS-KS because of its molecular mimicry of extracellular matrix molecules, which enhances the effects of angiogenic factors (3, 5, 19–22). These early-stage lesions have a polyclonal nature and can regress (23, 24). However, in time they can become monoclonal, especially in the nodular stage, and can evolve into a true sarcoma, probably in association with the increased expression of antiapoptotic oncogenes (25–28).

We and others have recently demonstrated by immunohistochemical analysis of evolutionarily staged lesions derived from AIDS-KS and classic KS patients that KS lesions have significant cytoplasmic levels of Bcl-2 (29, 30), a proto-oncogene known to prolong survival of quiescent nonproliferating cells by inhibiting the process of programmed cell death (31, 32). Bcl-2 was detected in endothelial and spindle cells of the lesions, and its expression was found to increase with lesion stage, reaching the maximal levels in nodular, late-stage lesions (29). Moreover, high Bcl-2 protein levels have been found to be associated with low levels of apoptosis (33), indicating that KS lesion progression is dependent on a dysregulation of apoptosis.

Paclitaxel, a microtubule-stabilizing agent, has been demonstrated in preclinical and clinical studies to be highly effective against several tumors, including ovarian, breast, and lung carcinomas (34). More recently, paclitaxel has been shown to be active in AIDS-KS patients in phase II clinical trials (35, 36), where it has been approved as a second line therapy after anthracyclines (37–40).

Paclitaxel has been shown to inhibit Bcl-2 antiapoptotic activity by phosphorylating the protein on a serine residue in the G_2-M

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[3] Abbreviations used in this paper: KS, Kaposi's sarcoma; IC, inflammatory cytokines; bFGF, basic fibroblast growth factor; HHV-8, human herpesvirus-8; KSC, KS spindle cells; APAAP, alkaline phosphatase-anti-alkaline phosphatase; ECGS, endothelial cell growth supplement.

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phase of the cell cycle, promoting apoptosis of acute leukemia, lymphoma, breast cancer, and prostate cancer cell lines (41–45). It has also been reported that Bcl-2 protein down-regulation results in induction of apoptosis in other in vitro systems like $CD34^+HLA\text{-}DR^+$ bone marrow cells after serum deprivation (46) and leukemia cells after ionizing radiation or arsenic-containing compound treatment (47–49) and in several tumors after treatment with Bcl-2 antisense oligodeoxynucleotides (50–54).

In this report we investigated the mechanism(s) of paclitaxel activity in KS by utilizing a model of experimental KS that is induced in nude mice by the s.c. inoculation of primary KS spindle cells (KSC) derived from human KS lesions (3, 4, 55). In this system KSC induce formation of angioproliferative lesions of mouse cell origin closely resembling human early KS (2–4). By using this model, we found that paclitaxel promotes regression of KS lesions in vivo. By in vitro studies we also demonstrated that it blocks the growth, migration, and invasion of KS spindle cells. Finally, paclitaxel treatment promoted apoptosis and down-regulated Bcl-2 protein expression in KSC in vitro and in KS-like lesions in mice.

Materials and Methods

Cell cultures

Primary spindle cell cultures derived from AIDS-KS lesions (KS6, KS8, and KS12 strains; passages 8–12) were derived and cultured as already described (2, 4, 6, 56, 57). Lesional spindle cells are latently infected by HHV-8 in vivo but lose the virus upon culture in vitro (58). Our primary KSC were found negative by PCR for HHV-8 DNA (our unpublished data).

Animal studies

To study the in vivo effect of paclitaxel on experimental KS, 4- to 6-wk-old nu/nu mice (CD1 background; Charles River Breeding Laboratories, Calco, Italy) were injected s.c. in the lower back with KSC (3×10^6) resuspended in 0.2 ml of 10% FCS RPMI 1640 and mixed with an equal volume of matrigel (Collaborative Biomedical Products, Bedford, MA) before inoculation (3, 4). On day 1 the mice were injected daily with 200 μg (10 mg/kg; two animals) or 500 μg (25 mg/kg; two animals) of paclitaxel (Taxol; Bristol-Myers Squibb, Princeton, NJ) in 0.5 ml of saline solution i.p. for 5 days (Expt. 1) or received only two doses (500 μg) on days 0 and 3 (Expt. 2). Control animals received saline solution i.p. The size of the lesions present at the injection site was evaluated daily by caliper measurement of the two major perpendicular diameters. On the sixth day, mice were sacrificed, and lesions were excised in toto and fixed in formalin. The histological features of the lesions were evaluated on cross-sections after staining with hematoxylin and eosin. To study Bcl-2 protein modulation by paclitaxel in vivo, nude mice were inoculated with KSC s.c. and treated with paclitaxel (two doses of 500 μg on days 0 and 2) or saline solution i.p. (three animals/group). Animals were sacrificed on day 4, and the lesions present at the injection sites were excised and frozen in OCT. Immunohistochemical analysis was performed on sections fixed with cold acetone using a rat anti-mouse Bcl-2 mAb (Santa Cruz Biotechnology, Santa Cruz, CA; 1:200 dilution) and a rat anti-mouse CD31 mAb (PharMingen, San Diego, CA; 1:500 dilution) by the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method as has been described (3). Briefly, sections were treated with rabbit serum to block nonspecific binding sites (Dako, Glostrup, Denmark; 1:10 dilution), and after incubation with the primary Ab, a rabbit anti-rat mAb (Dako; 1:50 dilution) was applied before a rat APAAP complex (Dako; 1:25 dilution). The negative control was performed by using an isotype mAb (rat IgG2a; PharMingen). All incubations were performed for 30 min. Bound primary Ab was detected with Fast Red chromogen (Dako). The slides were counterstained with Mayer's hematoxylin before evaluation. The percentage of positive cells in five high-power fields (×40 magnification) were counted and presented as the mean with the range of the readings. The care and use of mice were in accordance with the European Community guidelines.

KSC growth assay

For cell growth evaluation, KSC were plated in triplicate (2×10^3 cells/well in 12-well plates) in culture media containing endothelial cell growth supplement (ECGS) (Collaborative Biomedical Products) and were incubated with increasing concentrations of paclitaxel. After 3 days of culture, KSC were counted by trypan blue dye staining, as previously described (57).

TUNEL assay

KSC were cultured in eight-well chamber slides (Nunc, Naperville, IL; 4×10^4 cells/well) in KSC culture medium containing ECGS and increasing concentrations of paclitaxel for 24 h. The cells were then fixed in 80% ethanol/methanol (5 min each), and DNA strand breaks of apoptotic cells were identified in situ by a TUNEL assay kit (Boehringer Mannheim, Mannheim, Germany) following the manufacturer's instructions with some modifications. Briefly, 150 μl of TUNEL reaction mixture, containing TdT and fluoresceinated nucleotide mixture, was added to the wells (30 min, 37°C). Fluoresceinated nucleotides incorporated in polymers by the TdT-based enzymatic reaction were detected by immunohistochemical staining using a mouse anti-FITC mAb (Dako; 1:20 dilution) for 1 h at room temperature and by the APAAP method (3). The percentage of positive cells was determined as the mean of five high-power (×40 magnification) microscopic fields.

Apoptotic cell death assay

DNA fragmentation due to apoptotic death of KSC was quantitated by detection of cytoplasmic nucleosomes using the Cell Death Detection ELISA kit (Boehringer Mannheim) following the manufacturer's instructions. Briefly, KSC (5×10^4 cells/well in 1% FBS RPMI 1640 medium) were incubated in 12-well plates with increasing concentrations of paclitaxel for 3 days, and then both adherent and nonadherent cells were collected in microfuge tubes, washed, and lysed. The cytoplasmic fraction was recovered after centrifugation, and nucleosomes were assayed as recommended by the manufacturer. Each sample was tested in duplicate, and the results are expressed relative to the untreated control (apoptotic index).

Table 1. *Regression of KS-like lesions in vivo by paclitaxel*[a]

Expt.	Treatment	Mice Treated	Lesion Size at Sacrifice (mean cm^2)[b]	Regressing Area % (mean)[c]
1	Paclitaxel 200 μg (five doses)	2	0.6	42.5
	Paclitaxel 500 μg (five doses)	2	0.44	62.5
	Saline solution (five doses)	2	0.51	0.0
2	Paclitaxel 500 μg (two doses)	3	1.1	66.0
	Saline solution (two doses)	1	0.9	0.0

[a] Four- to 6-wk-old CD1 nu/nu mice were inoculated s.c. with KSC (3×10^6) to induce formation of KS-like lesions as described previously (4). Twenty-four hours later, the animals were injected i.p. daily for 5 days with 200 μg or 500 μg of paclitaxel (Expt. 1), or they received only two doses (500 μg) on days 0 and 3 (Expt. 2). Control animals were injected with saline solution. On day 6 the mice were sacrificed, and KS-like lesions present at the injection site were excised in toto and processed for histologic examination by hematoxylin-eosin staining of lesion cross-sections.

[b] Lesion area (cm^2) determined as described in *Materials and Methods* by caliper measurement of the two major perpendicular diameters.

[c] Percentage (%) of the regression area over the whole KS-like lesion area, characterized by necrotic tissue and cells with picnotic nuclei, inflammatory cell infiltration (macrophages and polymorphonucleated cells), and absence of vessels.

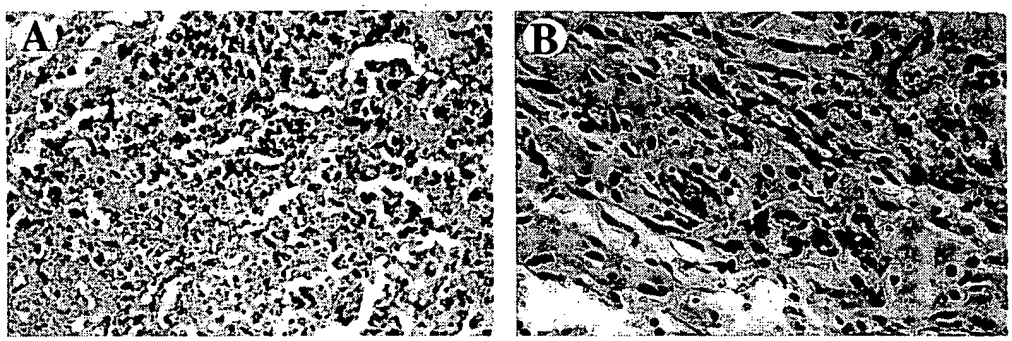

FIGURE 1. Paclitaxel induces regression of experimental KS lesions in nude mice. Nude mice bearing KS-like lesions were injected with paclitaxel or saline solution, as described in Table I. After 6 days of treatment, microscopic examination of hematoxylin-eosin-stained lesion cross-sections was performed. *A*, Lesions from paclitaxel-treated mice presented a central regressing area characterized by cells with picnotic nuclei, inflammatory cell infiltration (macrophages and polymorphonucleated cells), and absence of vessels surrounded by viable tissue rich in neoformed vessels and proliferating spindle cells. *B*, No signs of lesion regression were observed in control lesions.

KSC migration and invasion assay

Assays were performed as already described (59). Briefly, 10^5 KSC were plated in triplicate in the upper compartment of Boyden chambers in 0.1% BSA RPMI 1640 medium containing increasing concentrations of paclitaxel. Human recombinant bFGF (Boehringer Mannheim) diluted at 50 ng/ml in serum-free medium was placed in the lower compartment as chemoattractant. In the migration assays, the lower and upper compartments were separated by 12-mm-pore polycarbonate filters coated with collagen IV. In the invasion assays, filters were coated with collagen IV and matrigel, which prevents the migration of noninvasive cells. After 5 h (migration) or 6 h (invasion) of incubation, the cells remaining on the upper surface of the filters were mechanically removed, whereas cells migrated in the lower surface were fixed in methanol and stained with toluidine blue (Sigma, St. Louis, MO). Five to 10 random filter fields were counted by light microscopy as described previously (59). Assays were repeated three times.

Western blot analysis of Bcl-2 protein expression

KSC were cultured for 24 h with paclitaxel (100 nM), lysed in a modified RIPA buffer (150 nM sodium chloride, 50 nM Tris (pH7.5), 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 nM sodium orthovanadate, 50 mg/ml aprotinin, 50 mg/ml leupeptin, 1 mM PMSF, and 2 mM EGTA), and total proteins were extracted and measured by the Bradford assay (Bio-Rad, Hercules, CA) using BSA as standard. Equal amounts of proteins (140 μg) extracted from control- or drug-treated cells were subjected to 12% SDS-PAGE and then transferred onto nitrocellulose membrane (Amersham Life Science, Little Chalfont, U.K.) at 30 V overnight. Filters were then rinsed in blocking buffer (5% nonfat dry milk and 0.1% Tween 20 PBS) for 1 h at room temperature and subjected to immunoblotting using a mouse anti-Bcl-2 mAb (Dako; 1:50 dilution) for 1 h at room temperature and a HRP-conjugated goat anti-mouse secondary mAb (Amersham Life Science; 1:15,000 dilution) for 30 min. Membranes were then washed with 0.1% Tween 20 PBS, and detection was performed with the enhanced chemiluminescence method (ECL System; Amersham Life Science) by exposing blots to a Kodak XCL film (Eastman Kodak, New Haven, CT). Densitometry of specific and nonspecific bands was performed using an Imaging Densitometer GS-700 and a MultiAnalyst software (Bio-Rad). Because KSC are negative for HHV-8, we excluded any interference with viral Bcl-2 protein (60).

Results

Paclitaxel induces regression of KS-like lesions in vivo

We have previously demonstrated that injection of primary spindle cells of endothelial origin derived from human KS lesions (KSC) into nude mice induces transient angioproliferative lesions of mouse cell origin closely resembling early human KS (3, 4, 55). These lesions develop in response to cytokines such as bFGF and vascular endothelial growth factor released by KSC and are characterized by intense neoangiogenesis, spindle cell proliferation, and edema (2–4, 6, 8, 10, 11). The same molecules and histopathology are found in human primary lesions (3, 10, 61), suggesting that this in vivo model nicely reproduces KS in humans.

To assess the effect of paclitaxel in experimental KS in vivo, nude mice were inoculated s.c. with KSC. Beginning 24 h later the animals were injected daily for 5 days with paclitaxel 200 μg (10 mg/kg) or 500 μg (25 mg/kg) i.p. (Table I, Expt. 1). Control animals were injected with the same volume of diluent solution (saline solution). On day 6, the mice were sacrificed, and KS lesions were excised and examined histologically.

Lesion cross-sections from paclitaxel-treated mice showed a central regressing area characterized by cells with picnotic nuclei, inflammatory cell infiltration (macrophages and polymorphonucleated cells), and absence of vessels surrounded by viable tissue rich in neoformed vessels and proliferating spindle cells (Fig. 1*A*). No signs of lesion regression were observed in the control group (Fig. 1*B*). The mean regressing area was about 43% of the whole lesion in the animals treated with 200 μg of paclitaxel and increased up to 80% of the whole lesion (average, 62.5%) in the animals treated with 500 μg of paclitaxel (Table I, Expt. 1). However, the higher dose appeared to be toxic for the animals because it induced a

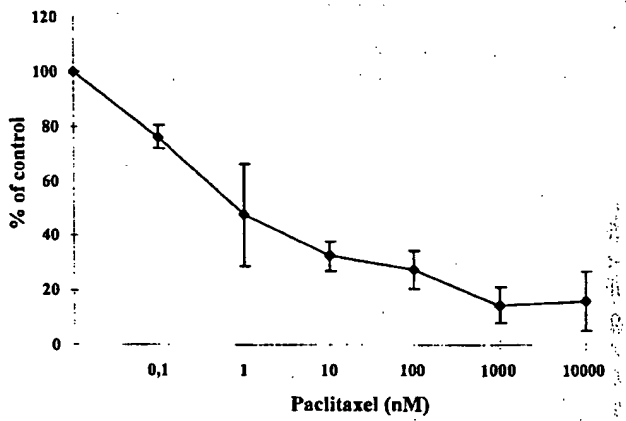

FIGURE 2. Paclitaxel inhibits KSC growth. KSC were plated in triplicate (2×10^3 cells/well, 12-well plates) in culture medium containing ECGS and were incubated with increasing concentrations of paclitaxel. After 3 days of culture, KSC were harvested and counted by trypan blue dye staining, as previously described (57). The mean percentage of cells (±SD) relative to the untreated control from three different experiments is shown.

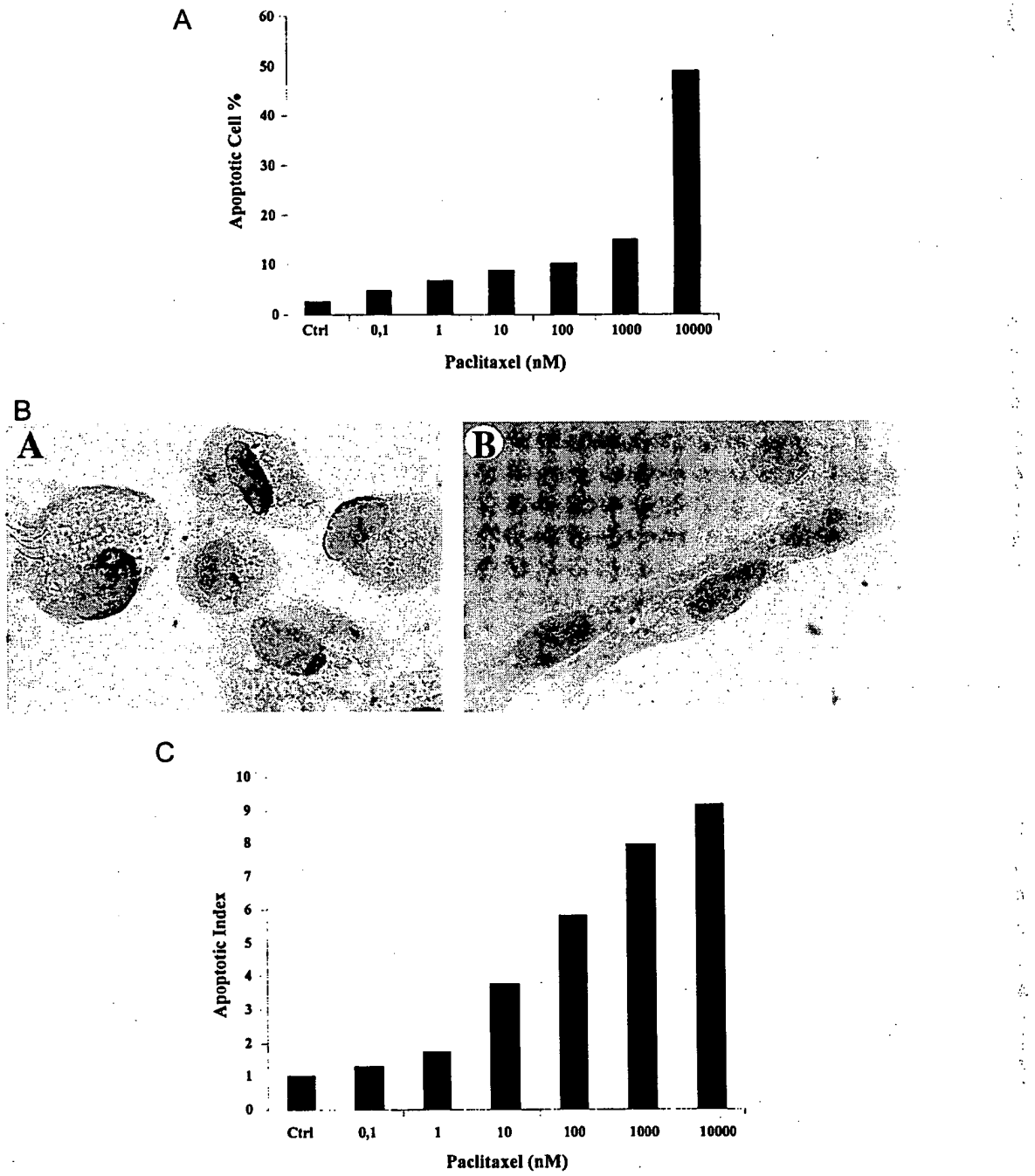

FIGURE 3. Paclitaxel induces apoptosis of KSC. KSC (4×10^4 cells/well in ECGS containing medium) were cultured for 24 h in chamber slides with increasing concentrations of paclitaxel as described in *Materials and Methods*. The cells in the chambers were then fixed, and DNA strand breaks of apoptotic cells were identified by TUNEL assay. *A*, The percentage of positive cells (average of positive cells in four high-power microscopic fields) from a representative experiment (of three performed) is presented. *B*, On the left (*A*), paclitaxel-treated apoptotic KSC positive for the TUNEL reaction are shown. KSC appear rounded and present the typical perinuclear localization of the staining. On the right (*B*), diluent-treated control KSC, negative for the TUNEL reaction, show the characteristic spindle morphology. *C*, DNA fragmentation due to apoptotic death of KSC was also determined by detection of cytoplasmic nucleosomes released during the apoptotic process. KSC (5×10^4 cells/well in 1% FCS RPMI 1640 medium) were incubated in 12-well plates with increasing concentrations of paclitaxel for 3 days, and then both adherent and nonadherent cells were collected and lysed. The cytoplasmic fraction was recovered, and the amount of released nucleosomes were assayed by an ELISA test, as described in *Materials and Methods*. Each sample was tested in duplicate, and the results are expressed relative to the untreated control (apoptotic index). A representative experiment of three performed is shown.

weight loss of 13% with respect to the control group, whereas the lower dose induced a 2% weight loss. In a confirmatory experiment, nude mice received 500 μg of paclitaxel i.p. twice, at 6 h and 3 days after KSC inoculation. In this case, the mean of the central regressing area was 66% of the whole lesion (range, 50–80%; Table I, Expt. 2) and no systemic toxicity (weight loss) was observed compared with the untreated animals (data not shown). Because the animals were examined only 6 days after the treatment was started (due to the transient nature of murine KS-like lesions), the histological signs of regression were not accompanied by a significant reduction of lesion size as measured at the time of sacrifice in treated vs control mice (Table I).

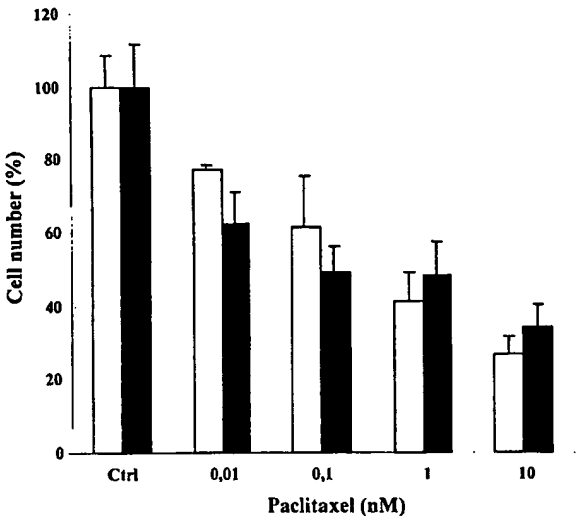

FIGURE 4. Paclitaxel inhibits the migration and invasion of KSC. KSC were plated in the upper compartment of Boyden chambers in a medium containing increasing concentration of paclitaxel (0.01 nM–10 nM), as described in *Materials and Methods*. The lower compartments, containing bFGF as chemoattractant, were separated by filters coated with collagen IV (migration) or collagen IV and matrigel (invasion). After 5 h (migration) or 6 h (invasion), cells migrated on the lower surface of filters were fixed, stained, and counted by light microscopy. The averages of migrating (□) and invading cells (■) from three different experiments are presented as the percentage of the untreated control cells, which was given a value of 100%. To exclude that the observed inhibition of migration and invasion promoted by paclitaxel was not due to an increase of cell death, two different strains of KSC (KS6 and KS12) have been incubated with paclitaxel at the highest dose used (10 ng/ml) and assayed for cell viability by FACS analysis after propidium iodide staining. After 6 h of incubation, no modifications of cell viability were observed in treated vs untreated KS cells (the ratio of propidium iodide-positive cells in treated vs untreated cells was 0.85 and 0.87 for KS6 and KS12 cell strains, respectively) (data not shown).

Paclitaxel inhibits growth and induces apoptosis of KSC

Experiments were then performed to study the mechanism of paclitaxel effects on KS. To this goal, the effect of paclitaxel was evaluated on KSC growth in vitro. As shown in Fig. 2, increasing concentrations of the drug inhibited KSC growth at concentrations as low as 0.1 nM with an IC_{50} around 0.1–1 nM and with a maximal effect at the highest dose tested (10 μM with 83.8% inhibition).

Because the inhibition of growth by paclitaxel was accompanied by an increase of cell death as measured by trypan blue dye staining, it was investigated whether the drug could promote apoptosis of KSC. To this goal, TUNEL assays were performed on paclitaxel-treated KSC. As shown in Fig. 3, *A* and *B*, a TUNEL test performed on KSC showed a gradual increase in the number of apoptotic cells after 24-h exposure to increasing concentrations of paclitaxel (0.1 nM–10 μM) that reached 49% of apoptotic cells at the highest dose tested (Fig. 3, *A* and *B*). The apoptotic DNA fragmentation observed in KSC after 3 days of treatment with increasing concentrations of paclitaxel (0.1 nM–10 μM) was confirmed by using an ELISA that detects cytoplasmic nucleosomes generated during the apoptotic cell death. As shown in Fig. 3*C*, paclitaxel treatment induced up to a 9-fold increase of apoptosis in KSC at the highest dose tested.

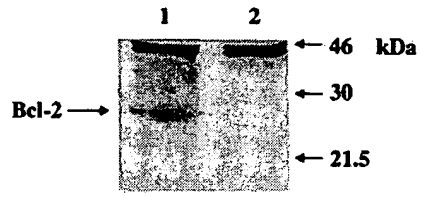

FIGURE 5. Paclitaxel inhibits Bcl-2 protein expression of KSC in vitro. KSC were cultured with 100 nM of paclitaxel for 24 h. Total protein extracts were prepared from control- or drug-treated cells, and equal amounts of proteins (140 μg) were resolved by 12% SDS-PAGE before immunoblotting with a mouse anti-Bcl-2 mAb. *Lane 1* corresponds to control KSC, and *lane 2* corresponds to paclitaxel-treated KSC (100 nM paclitaxel). Bcl-2 protein expression was 14-fold higher in untreated than in paclitaxel-treated KSC by densitometric analysis, whereas the signal detected from nonspecific bands (molecular mass, ~45 kDa) was about the same (ratio of *lane 1* vs *lane 2* = 1.09), confirming an equal loading of the two samples. To determine whether the inhibition of Bcl-2 expression by paclitaxel was specific for this protein, experiments were addressed to exclude that paclitaxel interfered with the expression of bFGF, a protein normally expressed in KSC. No down-regulation of bFGF expression was observed by ELISA upon normalization to total protein content or cell number (data not shown).

Paclitaxel blocks KSC migration and invasion

To determine whether paclitaxel interferes with the migrating and invasive properties of KSC in response to angiogenic factors, the drug (0.01–10 nM) was added to KSC placed in the upper compartment of Boyden chambers, whereas bFGF, used as chemoattractant, was placed in the lower compartment. Paclitaxel inhibited the migration of KSC and their invasion through a matrigel basal membrane (Fig. 4). These inhibitory effects were dose-dependent and occurred at doses as low as 0.01 nM, with an IC_{50} of 1 nM for cell migration and 0.1 nM for cell invasion, respectively. The inhibition on KSC invasion was generally more pronounced than on migration of these cells at all the doses tested (with the exception of 1 nM) (Fig. 4).

Paclitaxel down-regulates Bcl-2 expression in vitro and in vivo

Recently it has been demonstrated that at concentrations higher than those used in these experiments, paclitaxel induces Bcl-2 phosphorylation and apoptosis (41–45). Other groups have observed Bcl-2 protein down-regulation and apoptosis after serum deprivation or treatment with ionizing radiation, arsenic-containing compounds, or Bcl-2 antisense oligodeoxynucleotides (46–54). To assess whether the drug acted on KSC by interfering with the antiapoptotic oncogene Bcl-2, KSC were treated with 100 nM of paclitaxel for 24 h, and Bcl-2 protein levels were determined by Western blot analysis. As shown in Fig. 5, KSC expressed detectable levels of Bcl-2 protein, and treatment with paclitaxel markedly reduced its expression. However, no changes in the protein mobility due to phosphorylation were observed (Fig. 5). Because Bcl-2 phosphorylation has been reported to occur in the G_2-M phase of the cell cycle or after cell exposure to higher doses of paclitaxel (43, 45), KSC, which are slow-growing primary cultures with a doubling time of 48–72 h, were incubated with paclitaxel for 6–72 h or with higher drug concentrations (1–10 μM; data not shown). However, even under these experimental conditions, no phosphorylation of Bcl-2 was detected in KSC. Perhaps the low Bcl-2 levels present in primary KSC may preclude from detection of phosphorylation, as was already observed in other systems (62).

Table II. *Down-regulation of Bcl-2 protein expression in KS-like lesions by paclitaxel*[a]

Treatment	Mice Treated	Bcl-2 Positive Cells % Average (range)
Saline solution (two doses)	3	22.5 (14.4–32.1)
Paclitaxel 500 μg (two doses)	3	4.1 (1.2–7.3)

[a] Nude mice were inoculated with KSC s.c. and treated with paclitaxel (two doses of 500 μg on days 0 and 2) or saline solution i.p. (three animals/group), as described in Table I. Animals were sacrificed on day 4, and the lesions present at the injection sites were excised and frozen in OCT. Immunohistochemical analysis was performed on sections fixed with cold acetone using a rat anti-mouse Bcl-2 mAb by the APAAP method as described in *Materials and Methods*. The percentage of positive cells in five high-power fields (×40 magnification) was counted and presented as the mean with the range of the readings.

To determine whether the inhibition of Bcl-2 protein expression by paclitaxel was specific for this protein, experiments were addressed to exclude that paclitaxel interfered with the expression of other proteins normally expressed by KSC. To this goal, paclitaxel-treated KSC (at doses ranging from 1 μM to 0.1 nM for 48 h) were tested by ELISA for intracellular bFGF protein content, a protein produced at high levels by KSC (2, 8). No down-regulation of bFGF expression was observed upon normalization to total protein content or cell number (data not shown).

To determine whether paclitaxel treatment also promoted Bcl-2 down-regulation in our in vivo model of experimental KS, immunohistochemical analysis of Bcl-2 expression was performed in lesions from paclitaxel-treated animals vs untreated mice. Nude mice were inoculated with KSC s.c. and were treated with paclitaxel (two doses of 500 μg on days 0 and 2) or saline solution i.p. (three animals/group). Animals were sacrificed on day 4, and the lesions were analyzed for Bcl-2 expression, whereas CD31 expression was used as positive control. As shown in Table II and Fig. 6, paclitaxel treatment strongly inhibited Bcl-2 protein expression in the treated mice compared with the control group (4.1% of Bcl-2-positive cells vs 22.5%, respectively; shown in Fig. 6, *A* and *B*). Moreover, as already observed at the histological level in the previously described experiments, the periphery of the lesions from both untreated and treated mice showed the presence of many neoformed vessels and spindle-shaped cells positive for the CD31 endothelial cell marker (Fig. 6*C*). Thus, paclitaxel treatment down-regulates Bcl-2 protein expression both in vitro and in vivo.

Discussion

Paclitaxel is a drug highly effective in the treatment of several neoplasms, including ovarian, breast, and lung carcinomas (34). By affecting the microtubules and cellular vital processes in nonmitotic phases of the cell cycle, the drug inhibits the growth of either rapidly or slowly proliferating tumors (34). Recently, paclitaxel also has been found to be active in patients with advanced HIV-associated KS (35, 36), and it has been approved for AIDS-KS as a second line therapy after anthracycline-based regimens (37–40).

We investigated the mechanism(s) of paclitaxel efficacy in in vivo and in vitro systems of experimental KS. Our results indicate that paclitaxel is highly effective in promoting regression of KS-like lesions induced in the nude mouse model at doses (10–25 mg/kg) that have shown antitumor activity in preclinical studies in mice (63). The comparison of different paclitaxel doses (200 μg vs 500 μg i.p.) and schedules (daily treatment for 5 days starting 24 h after KS cell inoculation vs two doses only, 6 h and 3 days after KS cell injection) indicated that the higher dose (corresponding to 500 μg of paclitaxel) administered twice is as effective in inducing KS regression as the daily treatment with the same dose, with no sign of the systemic toxicity (weight loss) that was observed for the daily injection. In both groups of animals, the regressing area was extended up to 80% of the whole KS lesion area, with an average of 66 and 62.5%, respectively. It was usually central to the lesion and was characterized by cells with picnotic nuclei, infiltration of monocytic/macrophagic, and polymorphonucleated cells in the absence of vessels. The surrounding viable tissue was instead rich in neoformed vessels and proliferating spindle cells.

The effect of paclitaxel was also evaluated on KSC growth in vitro. The drug inhibited KSC growth in a dose-dependent fashion, with an IC_{50} around 0.1–1 nM, and promoted cell death by apoptosis as assessed by TUNEL test and by measuring the amount of nucleosomes released in the cytoplasm of treated cells.

We also determined that paclitaxel strongly interfered with the migrating and invasive properties of KSC in response to bFGF. The inhibitory effects were dose-dependent and occurred at doses as low as 0.01 nM of paclitaxel, with an IC_{50} corresponding to the concentration of 1 nM for cell migration and 0.1 nM for cell invasion, respectively.

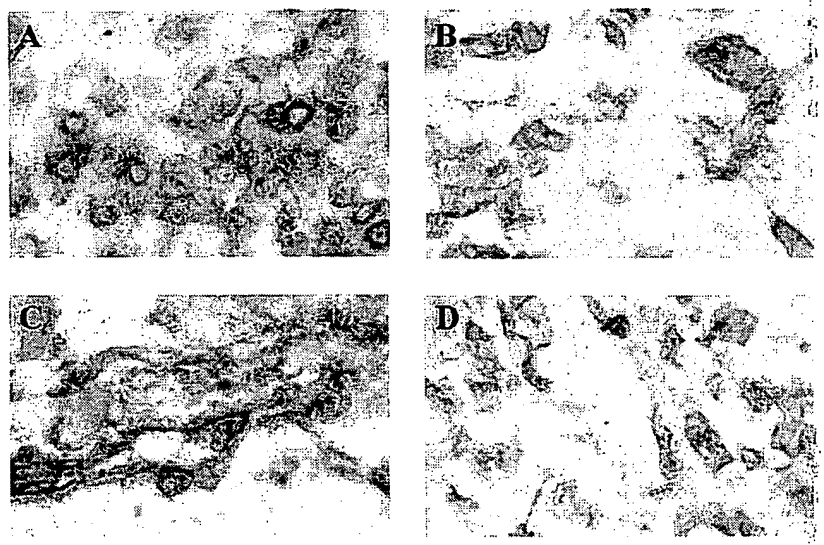

FIGURE 6. Paclitaxel inhibits Bcl-2 protein expression in murine KS-like lesions in vivo. Murine KS-like lesions were induced and treated as described in Table II. Immunohistochemical analysis of murine lesions was performed on frozen sections fixed with cold acetone using a rat anti-mouse Bcl-2 mAb by the APAAP method as described in *Materials and Methods*. Saline-treated lesions presented numerous Bcl-2-positive cells (*A*), whereas in paclitaxel-treated animals Bcl-2 protein expression was almost completely down-modulated (*B*) (×100 magnification). The periphery of the lesions from both untreated and treated mice showed the presence of many neoformed vessels positive for the CD31 endothelial cell marker (*C*) (×100 magnification). In *D*, a negative isotype control is shown.

Paclitaxel interaction with the cytoskeleton is well characterized and distinct from the binding sites of other microtubule-disrupting agents such as vinca alkaloids (64–66). In addition to inducing effects on interphase microtubule directly, several other activities of paclitaxel on cytoskeleton that may contribute to its cytotoxicity during nonmitotic phases of the cell cycle have been well characterized, including inhibition of chemotaxis, motility, invasion, and angiogenesis (65, 67–70). It is possible that paclitaxel exerts its activity in experimental KS through each of the above-mentioned mechanisms, as was also suggested by the observed effects of paclitaxel on the growth, migration, and invasion of KSC. However, it is noteworthy that, despite the reported effects on angiogenesis, we could observe at the lesion level, outside the central regressing area, an intense proliferation of neoformed vessels, suggesting a direct effect of the drug on lesional KSC.

Although the precise means by which cell death occurs are not clear, DNA fragmentation patterns characteristic of apoptosis have been documented after paclitaxel treatment of tumor cells (71, 72). Paclitaxel apoptotic effects have been associated with phosphorylation of Bcl-2, an antiapoptotic protein (41–45). In our experimental model, KSC expressed low but detectable levels of Bcl-2 protein, and treatment with 100 nM of paclitaxel for 24 h markedly reduced its expression. However, no protein phosphorylation was observed. Because Bcl-2 phosphorylation has been reported to occur in G_2-M phase of the cell cycle or after cell exposure to higher doses of paclitaxel and because KSC are slow-growing primary cultures (doubling time of 48–72 h) (43, 45), cells were incubated with paclitaxel for up to 72 h or with higher drug concentrations (1–10 μM). However, even under these experimental conditions, no phosphorylation of Bcl-2 was detected in KSC (data not shown). A possible explanation of this result is that the low Bcl-2 levels in KSC may preclude from detection of Bcl-2 phosphorylation, as already observed in other models (62). However, Bcl-2 protein down-regulation can also result in induction of apoptosis, as observed in other in vitro systems. Bcl-2 down-regulation and apoptosis have been observed in $CD34^+$ $HLA\text{-}DR^+$ bone marrow cells after serum deprivation (46), in childhood acute lymphoblastic leukemia cells after ionizing radiation (47), in acute promyelocytic leukemia cells after arsenic compound treatment (48, 49), and in several tumors after treatment with Bcl-2 antisense oligodeoxynucleotides, in which protein down-regulation was accompanied by an increased sensitivity to cytotoxic drugs (50–54). Thus, it is possible that paclitaxel interferes with KSC survival by down-regulating Bcl-2 antiapoptotic potential. Nevertheless, it should be mentioned that other non-Bcl-2-related mechanisms of survival might exist, as suggested by the resistance to cytotoxic drugs reported in some low Bcl-2-expressing lines (73, 74), possibly involving other Bcl-2 family members or signals derived from surrounding microenvironment (75).

The down-regulation of Bcl-2 protein expression by paclitaxel treatment has also been observed in our in vivo model of experimental KS. As demonstrated by immunohistochemical analysis, paclitaxel treatment strongly inhibited Bcl-2 protein expression in KS-like lesions induced in nude mice by the inoculation of KSC.

HHV-8, the recently identified herpesvirus found to be present in all forms of KS, encodes for a Bcl-2 homolog (76, 77) that has been demonstrated to inhibit Bax-mediated apoptosis (77). However, because HHV-8 is lost by KSC upon culture in vitro at very early passages, v-Bcl-2 cannot have a role in our experimental system. Similarly, it cannot have a relevance in paclitaxel-treated patients. In fact, this is a lytic gene expressed in very few spindle cells or infiltrating lymphomonocytes of KS lesions that are committed to die due to lytic viral infection, whereas the vast majority of spindle cells is latently infected and does not express v-Bcl-2 (76, 78).

The observation that paclitaxel may induce KS regression through Bcl-2 down-regulation is of particular interest because it supports previous data of a role of Bcl-2 in KS lesion formation and progression (29, 33). Bcl-2 up-regulation in KS lesions coupled with cell growth stimuli may divert cells from apoptosis toward continuous cell proliferation, and this may result in the transformation of reactive KS lesions into a true sarcoma. Paclitaxel, because of its activity on Bcl-2, may represent a pathogenetic approach to late-stage KS, when it progresses to a nodular form of monoclonal nature (25–28) that is usually refractory to conventional therapies.

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